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Involvement of *CIF1* (*GGS1/TPS1*) in osmotic stress response in *Saccharomyces cerevisiae*

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Abstract **The transcriptional responses of the osmotically induced genes *ALD2*, *CTT1*, *ENA1*, *GPD1*, *HSP12* and *HSP104*, were studied in *Saccharomyces cerevisiae* strains differing in *CIF1* gene function following application of osmotic stress. The *CIF1* gene (allelic to *GGS1* and *TPS1*) encodes a subunit of the trehalose synthase complex that affects trehalose synthesis. Recent work has implicated this gene in various signalling events in the cell, including transcriptional response to heat-shock treatment. Because many genetic factors can influence *S. cerevisiae* osmoresponse, we have compared the expression of osmotically induced genes and glycerol production in isogenic strains differing only in functionality of *CIF1*, growing logarithmically on galactose medium. When cultures were exposed to 0.8 M NaCl or 1.5 M sorbitol the *cif1* strain showed greatly reduced transcription of osmotically induced genes compared to the wild type. These treatments did not affect viability of the yeast strains. Treatment with 0.3 M NaCl produced no significant differences in transcription of these genes in *CIF1* or *cif1* strains. Treatment with 0.6 M sorbitol induced small but reproducible differences, with gene expression higher in the *CIF1* strain compared to the *cif1* mutant. When cultures were treated with 0.3 M NaCl or 0.6 M sorbitol for 1 h, glycerol production was similar for both strains, but after 3 h of the same treatment, total glycerol production was higher in the *CIF1* strain. When cultures were treated with 0.8 M NaCl for 3 h, the wild type strain produced more glycerol than the mutant strain. Both strains produced similar amounts of glycerol following exposure to 1.5 M sorbitol for 3 h, although the wild type strain showed enhanced ability to retain glycerol inside the cell. The results are discussed in the context of the possible role that the *CIF1* gene product has in response to osmotic stress.**

Key words: *Saccharomyces cerevisiae*; Osmotic shock; Glycerol; *CIF1*(*GGS1/TPS1*)

1. Introduction

When *Saccharomyces cerevisiae* is exposed to hyperosmotic stress, a number of physiological changes take place. These include; efflux of intracellular H_2O, rapid reduction in total cell volume, including the vacuole [1], a transient increase in glycolytic intermediates [2], and the eventual accumulation of glycerol in the cytosol [3]. Hyperosmotic stress also triggers the HOG (*H*yperosmotic *g*lycerol) signalling pathway. This results in the transcription of genes including *GPD1* [4], *ALD2* [5], *CTT1*, *HSP104* [6], *HSP12*, and *ENA1* [7]. However, gene transcription and osmotolerance are not limited to HOG-mediated events. Protein phosphatase 2B (calcineurin) has been implicated in regulating gene expression and osmotic tolerance [8,9]. Members of the *HAL* gene family appear to be involved as factors that will affect tolerance to increased osmolarities [10,11]. Further complexity of stress tolerance is shown in cross protection, or acquisition of tolerance to a particular stress condition by an apparently unrelated mild stress treatment. In the case of *S. cerevisiae*, heat-shock can protect against freezing, and heat tolerance can be obtained by osmotic stress and vice-versa [12–15]. In addition, the heat inducible genes *HSP104*, *CTT1*, *HSP12* and *HSP26* are strongly induced by osmoshock [6,15]. Other common aspects of heat and osmotic shock responses can be found with *MSN2* and *MSN4* gene function. These genes encode zinc-finger proteins that specifically bind to stress response elements (STREs) [16,17]. STREs are present in a large number of genes induced by heat or osmotic stress. Mutant *msn2/msn4* strains have reduced transcription of genes containing STREs, and have increased sensitivity to heat, osmotic and oxidative shocks and carbon source starvation [16,17]. The *YAP1* gene, a transcriptional activator implicated in drug resistance, has been found to activate sequences containing STREs [18]. In addition, the *ROX1* gene has been found to be involved in heat and osmoshock response. This involvement may be through STRE-like elements located upstream of the heat and osmotically induced *CYC7* gene [19]. Thus, acquisition of stress tolerance is complex, and likely to be the sum of responses from differing genes and pathways.

The *CIF1* gene of *S. cerevisiae* encodes a protein of the trehalose synthase complex. Trehalose has been shown to act as a protectant against extreme heating in cells cultured in non-fermenting conditions [20,21], and in high gravity fermentation [22]. Trehalose has been observed to accumulate in cells that have been osmotically stressed [2,23]. Mutants deficient in *CIF1* function are unable to accumulate trehalose and cannot grow using glucose, fructose or mannose as carbon sources. The *CIF1* gene is crucial for glucose induced regulatory events including transient increases of intracellular cAMP, induction of glycolytic enzymes, inactivation of gluconeogenic enzymes, activation of cation transport and stimulation of H^+-ATPase [24–26]. When *cif1* mutants growing in logarithmic phase in galactose cultures were heat shocked, they showed greatly reduced transcription of a number of heat inducible genes [27]. Given that the *CIF1* gene appears to be involved in a wide range of cellular responses, we have analysed expression of osmotically induced genes in strains differing only in *CIF1* gene function to determine if this gene affects hyperosmotic response.

2. Materials and methods

2.1. Yeast strains, growth and stress conditions

S. cerevisiae strains W303-1A (MAT*a*, *ade2*, *his3* *ura3*, *leu2*, *trp1*,

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CIF1) and WDC-3A (isogenic except for *cif1::HIS3*) [28] were used in this study. Strains were grown for up to 8 h to mid-logarithmic phase (approx. $2–3\times10^7$ cells/ml) at 25°C in GalYP broth as described previously [29]. For osmotic shock, 0.3 M or 0.8 M NaCl, or 0.6 M or 1.5 M sorbitol were added to these cultures(final concentration), which were incubated for a further 15 min or 1 h at 25°C.

2.2. Extraction of total RNA and mRNA analyses

Total RNA was extracted from control or stressed cells as described previously [30]. Approximately 50 μg of total RNA for each sample was loaded and run in denaturing gels containing 1.2% w/v agarose and 2.2 M formaldehyde [31]. Gels were blotted onto nylon membranes as described previously [27]. The gene probes *ALD2*, *ENA1*, *GPD1*, and *HSP12* were kindly supplied by Dr T. Hirayama [7]. *ACT1*, *CTT1* and *HSP104* have been described previously [27]. DNA for hybridisation was labelled with [α-^{32}P]dCTP using a Prime-it RmT labelling kit (Stratagene) following the manufacturers protocol. Hybridisation at 42–47°C and washing conditions have been described previously [31,32]. Pre-flashed X-ray film (Amersham Hyperfilm-MP) was exposed to post-hybridised membranes at −80°C for 16–48 h Sizes of transcripts were obtained by reference to the 25S (3395 nt) and 18S (1800 nt) ribosomal RNA bands in ethidium bromide stained gels. Results presented are typical of at least two cultures tested in duplicate.

2.3. Measurement of osmolality

Following the harvesting of cultures for RNA extraction, a portion of the supernatants was retained. Osmolality was measured by freezing point depression using an Advanced osmometer (Model 3D3) (Advanced Instruments).

2.4. Glycerol determination

For total glycerol determination, a 2 ml sample was removed from the culture and immediately boiled for 5 min. Cell debris was removed by centrifugation at $400\times g$ for 5 min at room temperature. External glycerol samples were prepared by filtering 5 ml of non-boiled culture through Alltech Nylon 66 membranes (pore size 0.2 micron diameter) attached to a vacuum manifold. The filtrate was boiled for 5 min and retained for assay. The assay for glycerol was carried out by reacting 100 μl of sample in a final volume of 1 ml. This contained 50 mM Tris (pH 8), 2.5 mM phosphoenolpyruvate, 1.25 mM ATP, 1.25 mM $MgSO_4$, 200 μM NADH, 5 U lactate dehydrogenase, 4 U pyruvate kinase and 1 U glycerokinase. The samples were incubated at 30°C for 30 min. The difference in A_{340} between samples and controls lacking glycerokinase were measured by spectrophotometer.

2.5. Determination of glycerol-3-phosphate dehydrogenase activity

Extracts were made from cell pellets prepared from 100 ml of culture. These were prepared as described previously [33] except the desalting step was omitted. Samples were assayed immediately following centrifugation. Assays were carried out as previously reported [34] in stirred 4 ml cuvettes using a Cary 3-E double-beam spectrophotometer. Protein levels were determined using a kit (Bio-Rad) based on the Bradford assay method.

3. Results and discussion

CIF1 and *cif1* strains have been shown to have key regulatory differences, including in cAMP mediated events and heat shock induction of genes [24–27]. Given that there are common elements of both heat and osmotic stress response, *CIF1* gene function may also affect the latter. The osmotic stress treatments produced the following media osmolality (expressed as milliosmols); no additives, 194; 0.3 M NaCl, 767; 0.6 M sorbitol, 852; 0.8 M NaCl, 1732; 1.5 M sorbitol, 1662. This allowed us to observe if moderate and high osmostress response occurred as a result of ionic stress (NaCl treatment) or a general osmotic stress (sorbitol). To ensure that osmostress treatments did not kill cultures used in this study, viable counts were carried out throughout the work. The osmotic stresses used produced no significant differences in viability between wild-type and mutant strains. Therefore, if differences were found to arise between the *CIF1* or *cif1* strains, it should be a result of genetic and/or physiological responses rather than cell death.

Following treatment with 0.3 M NaCl, both *CIF1* and *cif1* strains showed strong induction of all genes after 15 min (Fig. 1, lanes 1–4). After 1 h transcriptional activity was generally lowered, but the *cif1* strain showed higher gene transcription than the wild-type (Fig. 1, lanes 5 and 6) when results were compared to *ACT1* expression. This suggests that while mild salt stress is more transitory in W303-1A (*CIF1*), both strains respond to it. Experiments by other workers using *hog1* mutants showed that the transcriptional response was essentially abolished following 0.3 M NaCl treatment [6]. As *CIF1* and *cif1* strains showed similar initial responses following 0.3 M NaCl treatment, it appears that *CIF1* is not required to interact with the HOG pathway under these conditions. Internal and total glycerol levels were very similar for both strains after 1 h incubation in 0.3 M NaCl (Table 1). However after 3 h, significant differences were obtained. Over this interval, the *CIF1* strain produced more glycerol than the mutant, although the ratio of internal glycerol remained at similar levels for both strains. When both transcription and glycerol production are considered together it would appear that W303-1A (*CIF1*) showed a more rapid physiological adjustment to the conditions compared to WDC-3A (*cif1*), which had higher levels of transcription after 1 h 0.3 M NaCl treatment and lower total glycerol production after 3 h.

Following treatment with 0.6 M sorbitol, the *CIF1* strain

Table 1
Glycerol and production and retention by osmotically stressed cultures of *S. cerevisiae*

	W303-1A (*CIF1*)		WDC-3A (Δ*cif1::HIS3*)	
	Int glycerol	Total glycerol	Int glycerol	Total glycerol
Control	< 20	325	< 20	384
0.3 M NaCl 1 h	161 (35)	454	166 (31)	519
0.3 M NaCl 3 h	367 (41)	883	243 (40)	598
0.6 M sorbitol 1 h	130 (24)	541	86 (15)	560
0.6 M sorbitol 3 h	331 (36)	925	70 (12)	559
0.8 M NaCl 3 h	286	715	< 20	474
1.5 M sorbitol 3 h	66	706	< 20	746

Numbers express nanomoles of glycerol per 10^7 cells in shake flask cultures. Those in parentheses show the percentage of total glycerol retained by the cells in the cultures. The results shown are the means of three to five experiments carried out separately. Control samples were galactose grown cultures with no NaCl or sorbitol addition.

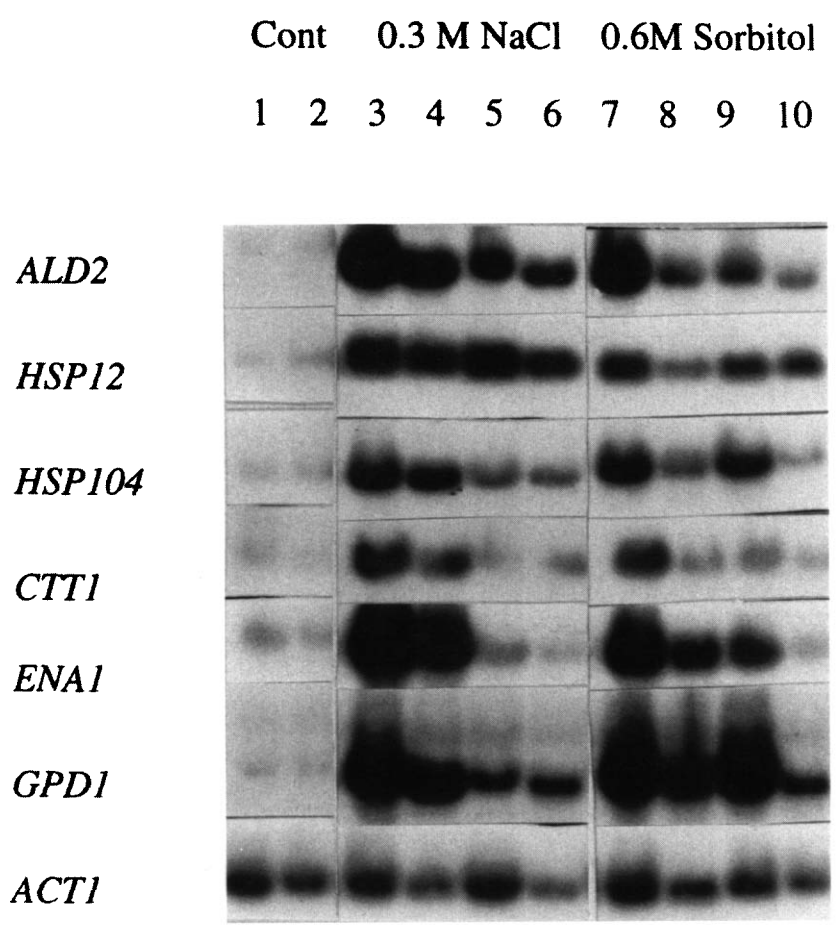

Fig. 1. Transcription of osmo-induced genes before and after moderate osmoshock in *S. cerevisiae* strains with functional or deleted *CIF1* activity. Strains W303-1A (*CIF1*) and WDC-3A (*cif1::HIS3*) were grown to early-log phase in galactose YP and exposed to either 0.3 M NaCl or 0.6 M sorbitol for 15 or 1 h. Total mRNA was probed separately for *ACT1* or other specific mRNA transcripts. Lane 1, W303-1A no treatment; lane 2, WDC-3A no treatment; lane 3, W303-1A 0.3 M NaCl 15 min; lane 4, WDC-3A 0.3 M NaCl 15 min; lane 5, W303-1A 0.3 M NaCl 1 h; lane 6, WDC-3A 0.3 M NaCl 1 h; lane 7, W303-1A 0.6 M sorbitol 15 min; lane 8, WDC-3A 0.3 M sorbitol 15 min; lane 9, W303-1A 0.6 M sorbitol 1 h; lane 10, WDC-3A 0.6 M sorbitol 1 h.

appeared to have higher transcription than the mutant after 15 min (Fig. 1, lanes 7 and 8). After 1 h this difference was generally maintained (Fig. 1, lanes 9 and 10). The results suggest that differing stress responses occur following ionic or nonionic stress treatment. The ionic (NaCl) response showed no reliance on *CIF1* function, but the nonionic (sorbitol) response had limited *CIF1*-dependence. Despite differences in transcriptional response, both strains produced similar amounts of glycerol after 1 h of treatmentwith 0.6 M sorbitol (Table 1). When this was extended to 3 h W303-1A (*CIF1*) continued to produce glycerol, while WDC-3A (*cif1*) showed no further increase. Under these conditions, WDC-3A did not retain internal glycerol level to the same degree as W303-1A. Recent findings presented by Sutherland et al. [34], indicated that sugar alcohols inhibited the action of a regulated glycerol facilitator encoded by the *FPS1* gene [35]. If this is the case, it would be expected that both strains would be unable to retain glycerol in the presence of sorbitol. Another possibility is that the *CIF1* yeast may have altered the composition of the plasma membrane to reduce leakage of glycerol under these conditions. Glycerol has been observed to passively diffuse through the membranes [35] and when yeast cultures are grown in the presence of 0.5 M NaCl significant alterations occur in the composition of the plasma membrane [36]. Other results using yeast treated with 0.4 M sorbitol elicited transcriptional response for osmo-responsive genes [37]. Combining these results with the observation that WDC-3A (*cif1*) showed a reduced transcriptional response (particularly over 1 h) compared to the wild type, it is possible that WDC-3A is slower to adapt its overall osmoticc response including changes in membrane composition, to counterbalance the 0.6 M sorbitol stress, and thus cannot retain synthesised glycerol. This may explain why W303-1A (*CIF1*) retained a smaller proportion of glycerol after 1 h incubation in the 0.6 M sorbitol compared to the 3 h treatment. In turn, this implies that *CIF1* is required to retain glycerol in response to sorbitol, but not salt at moderate osmotic pressures.

After stress treatment in the presence of 0.8 M NaCl W303-1A (*CIF1*) showed large increases in transcription of all stress responsive genes studied while WDC-3A (*cif1*), had greatly reduced transcriptional response (Fig. 2, lanes 3–6). Under these conditions transcriptional response appears to require *CIF1* function. When glycerol production and retention was assayed, WDC-3A (*cif1*) had reduced levels of internal and total glycerol after 3 h incubation in 0.8 M NaCl, relative to the wild-type strain (Table 1). This may be a result of

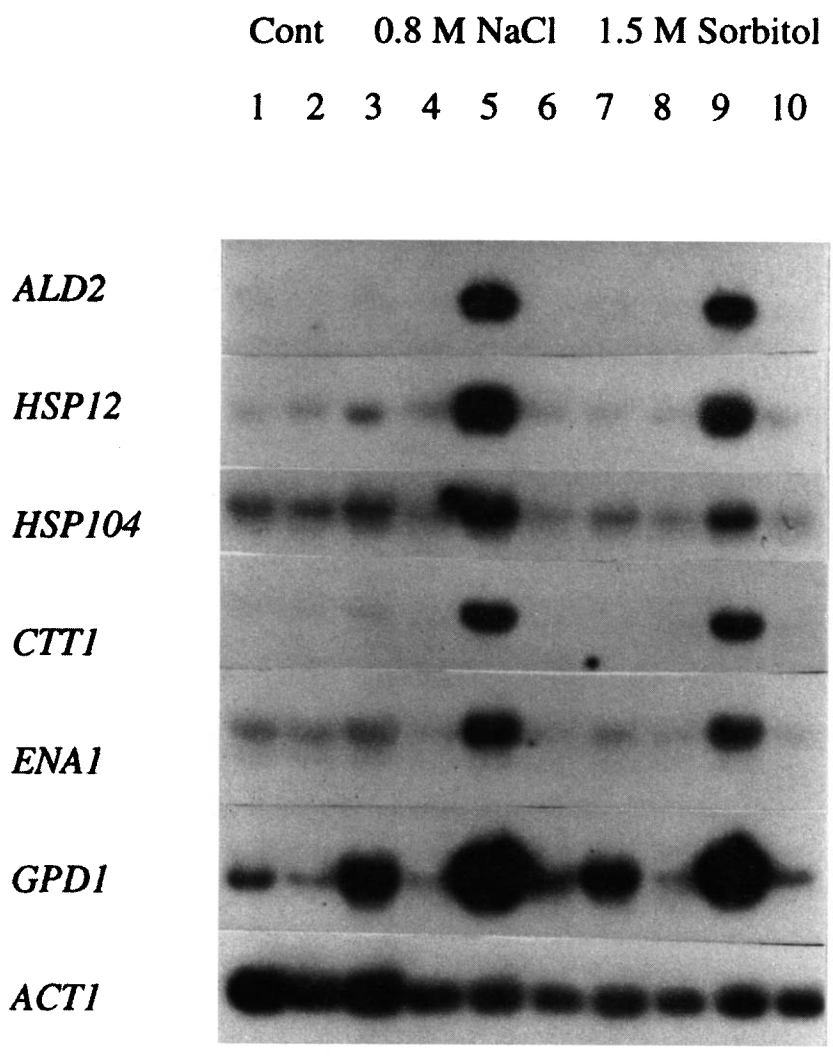

Fig. 2. Transcription of osmo-induced genes before and after high level osmoshock in *S. cerevisiae* strains with functional or deleted *CIF1* activity. Strains W303-1A (*CIF1*) and WDC-3A (*cif1::HIS3*) were grown to early-log phase in galactose YP and exposed to either 0.8 M NaCl or 1.5 M sorbitol for 15 or 1 h. Total mRNA was probed separately for *ACT1* or other specific mRNA transcripts. Lane 1, W303-1A no treatment; lane 2, WDC-3A no treatment; lane 3, W303-1A 0.8 M NaCl 15 min; lane 4, WDC-3A 0.8 M NaCl 15 min; lane 5, W303-1A 0.8 M NaCl 1 h; lane 6, WDC-3A 0.8 M NaCl 1 h; lane 7, W303-1A 1.5 M sorbitol 15 min; lane 8, WDC-3A 1.5 M sorbitol 15 min; lane 9, W303-1A 1.5 M sorbitol 1 h; lane 10, WDC-3A 1.5 M sorbitol 1 h.

reduced ability to adjust to the salt treatment compared to W303-1A (*CIF1*). Northern analysis of the *ENA1* gene supports this postulate. The *ENA1* gene encodes a Li^+, Na^+, K^+ ATP-ase which exports these ions out of the cell [38]. Since WDC-3A (*cif1*) cannot significantly increase levels of Ena1p to remove intracellular Na^+, other cellular processes such as glycerol production may be compromised.

When incubated in 1.5 M sorbitol the strains showed a similar transcriptional response to the 0.8 M NaCl stress treatment (Fig. 2, lanes 7–10). However, when glycerol production was tested, both strains produced similar amounts. While W303-1A (*CIF1*) could retain a small proportion of glycerol, WDC-3A (*cif1*) could not (Table 1). Since sorbitol is not permeable to *S. cerevisiae* (unpublished observations of this laboratory), it does not have to be excreted from the cell. Therefore, internal cell processes should not be affected so severely when compared to the 0.8 M NaCl treatment. However, given the transcriptional responses of both strains it would be expected that WDC-3A (*cif1*) would have reduced levels of glycerol compared to the wild-type. To test if levels of glycerol-3-phosphate dehydrogenase changed after osmoshock in 1.5 M sorbitol for 3 h, cell extracts were prepared and assayed. Strain W303-1A (*CIF1*) showed a 60-fold increase in enzyme levels while WDC-3A (*cif1*) had a 2.5-fold increase (Table 2). Although these results support the data

Table 2
Activities of glycerol-3-phosphate dehydrogenase in control and osmotically stressed cultures of *S. cerevisiae*

	W303-1A (*CIF1*)	WDC-3A (*Δcif1::HIS3*)
Control	1.94	2.77
1.5 M sorbitol 3 h	127	7.04

Values obtained for glycerol-3-phosphate dehydrogenase enzyme activity are expressed as nmoles glycerol-3-phosphate formed/min/mg protein. The results shown are the means of duplicate readings taken from three seperate cultures.

obtained by northern blots, it appears that levels of enzyme are not limiting glycerol production under these conditions [23]. It may be possible that both strains are limited for precursor metabolites or cofactors under these conditions. While other workers have found increases in the levels of glycerol-3-phosphate dehydrogenase and glycerol following osmoshock [4,33], the cultures used were glucose grown. Growth in glucose for *S. cerevisiae* produces sufficient levels of metabolites such as NADH and dihydroxyacetone phosphate for optimal glycerol production. In this work cultures were galactose-grown (*cif1* strains cannot grow using glucose as a carbon source). When yeast is grown using galactose as a carbon source, catabolite repression does not occur as with glucose, and sugar is metabolised more slowly, implying that the energy metabolism of *S. cerevisiae* using galactose is largely respiratory [39]. Yeast cultures growing by respiratory metabolism would have less dihydroxyacetone phosphate and NADH available for glycerol synthesis and so enzyme levels may not be limiting under these conditions. Hence both strains were able to produce similar amounts of glycerol during osmostress in 1.5 M sorbitol.

How *CIF1* affects response to osmotic stress is unclear. Activation of genes by applied stress seems to require specific sequences upstream of known stress response genes. In the case of heat-shock response, *CIF1* function appears to be important for transcription of heat responsive genes [27]. When cultures are stressed, *CIF1* appears to strongly influence transcription when osmoticant concentrations are high At lower concentrations of NaCl and sorbitol, this influence is either not observable or is less significant. One possibility is that *CIF1* may ultimately enhance the transcriptional activity of *MSN2/MSN4* or other known/unknown transcriptional factors may play a role with *CIF1* in mediating stress response. Results published by Miralles and Serrano [5] showed that the *ALD2* gene retained limited osmoresponse in a *Δhog1* genetic background. When the promoter of *ALD2* was fused to a *lacZ* reporter gene, osmoinduction of beta-galactosidase appeared to be unaffected when all STREs were deleted from the construct. This raises the likelihood that there are as yet, undiscovered transcriptional factors or enhancers that respond to osmotic stress. If *CIF1* function affects such factors, it appears that this effect is highly significant at increased solute concentrations. However, there is no published evidence that Cif1p binds to DNA.

The results in this work also show that *CIF1* function appears to affect the ability of yeast to retain glycerol following non-ionic osmostress with sorbitol, indicating a broader influence in physiological response other than transcriptional events. These may include the capability to alter membrane composition or characteristics to compensate for sorbitol stress, including the ability to retain synthesised glycerol. The *FPS1* gene (characterised as a facilitator for glycerol transport [35]) was originally isolated as a multicopy supressor of a *cif1* (*ggs1/tps1*) deletion [40] suggesting an interaction between these, at least at the physiological level. Deletion of *FPS1* has been reported to alter the composition of the membrane [34]. In a *cif1* background, *FPS1* may act to compensate for cell membrane changes. Although the role of *CIF1* in osmotic stress response has yet to be fully elucidated, this work highlights both the complexity of stress response in *S. cerevisiae* and important role that *CIF1* plays in the physiology of yeast.

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